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Analysis of acetylcholine receptor phosphorylation sites using antibodies to synthetic peptides and monoclonal antibodies

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Three peptides corresponding to residues 354–367, 364–374, 373–387 of the acetylcholine receptor (AChR) δ subunit were synthesized. These peptides represent the proposed phosphorylation sites of the cAMP-dependent protein kinase, the tyrosine-specific protein kinase and the calcium/phospholipid-dependent protein kinase respectively. Using these peptides as substrates for phosphorylation by the catalytic subunit of cAMP-dependent protein kinase it was shown that only peptides 354–367 was phosphorylated whereas the other two were not. These results verify the location of the cAMP-dependent protein kinase phosphorylation site within the AChR δ subunit. Antibodies elicited against these peptides reacted with the δ subunit. The antipeptide antibodies and two monoclonal antibodies (7F2, 5.46) specific for the δ subunit were tested for their binding to non-phosphorylated receptor and to receptor phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Antibodies to peptide 354–367 were found to react preferentially with non-phosphorylated receptor whereas the two other anti-peptide antibodies bound equally to phosphorylated and non-phosphorylated receptors. Monoclonal antibody 7F2 reacted preferentially with the phosphorylated form of the receptor whereas monoclonal antibody 5.46 did not distinguish between the two forms.

Key words: acetylcholine receptor/antibodies/phosphorylation/synthetic peptides

Introduction

The acetylcholine receptor (AChR) is a neurotransmitter-regulated ion channel which mediates synaptic transmission at the post-synaptic membrane of the neuromuscular junction (for review, see Popot and Changeux, 1984). The purified AChR is a 250 kd membrane protein which consists of four subunits with a stoichiometry of $\alpha_2\beta\gamma\delta$ and has been demonstrated to be a phosphoprotein *in vivo* (Vandlen *et al.*, 1979). Post-synaptic membranes rich in the AChR were found to contain endogenous protein kinases (Gordon *et al.*, 1977; Teichberg *et al.*, 1977; Saitoh and Changeux, 1981) as well as protein phosphatases (Gordon *et al.*, 1979). It was shown that post-synaptic membranes contain at least two endogenous protein kinases that phosphorylate the receptor. These include a cAMP-dependent protein kinase (Huganir and Greengard, 1983; Zavoico *et al.*, 1984), and a tyrosine-specific protein kinase (Huganir *et al.*, 1984). Furthermore, a calcium/phospholipid dependent protein kinase has also been suggested to be involved in AChR phosphorylation (Huganir *et al.*, 1983). More recently, it was demonstrated that phosphorylation of AChR by cAMP-dependent protein kinase increases its rate of rapid desensitization (Huganir *et al.*, 1986). These results provide the first direct evidence that phosphorylation of the receptor modulates its function.

Based on the amino acid sequences of the four AChR subunits (Noda *et al.*, 1982, 1983a,b; Claudio *et al.*, 1983) and considering the subunit specificity of the protein kinases and their substrate requirements, possible phosphorylation sites for the three protein kinases were proposed (Huganir *et al.*, 1984).

Among the four receptor subunits, the δ subunit was found to be phosphorylated to the highest extent (Vandlen *et al.*, 1979; Huganir *et al.*, 1984). We therefore set out to localize the different phosphorylation sites on this subunit using a synthetic approach. We have already reported our first study on the mapping of the cAMP-dependent phosphorylation sites on AChR (Souroujon *et al.*, 1986a). Synthetic peptides and their antibodies have also been employed in our laboratory for mapping the cholinergic binding site on the α subunit of AChR (Neumann *et al.*, 1985, 1986). This approach has also been used to study the phosphorylation of other receptors (Herrera *et al.*, 1985; Gullick *et al.*, 1985) and phosphoproteins (Nairn *et al.*, 1982). In the present study we have employed three synthetic peptides and their respective antibodies for analysis of the different proposed phosphorylation sites on the δ subunit of AChR.

Results

Peptide phosphorylation

Three peptides corresponding to residues 354–367 (Souroujon *et al.*, 1986a), 364–374 and 373–387 of the δ subunit of *Torpedo* AChR were synthesized. These peptides include the proposed sites of phosphorylation by the cAMP-dependent protein kinase, the tyrosine-specific protein kinase and the calcium/phospholipid-dependent protein kinase respectively. The sequences of the three synthetic peptides are shown in Figure 1. These sequences are located in a cytoplasmic domain of the δ subunit according to all the models which predict the transmembrane orientation of the AChR molecule (Noda *et al.*, 1983b; Devillers-Thiery *et al.*, 1983; Claudio *et al.*, 1983; Finer-Moore and Stroud, 1984; Guy, 1984).

All three peptides were tested for their ability to undergo phosphorylation *in vitro* by the catalytic subunit (C-sub) of the cAMP-dependent protein kinase. Phosphorylation products were

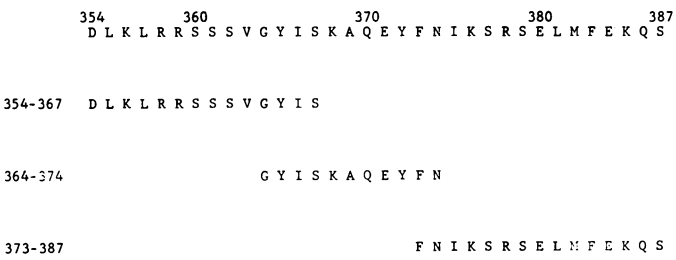

Fig. 1. Amino acid sequences of three synthetic peptides corresponding to residues 354–367, 364–374 and 373–387 of the δ subunit of *T. californica* AChR.

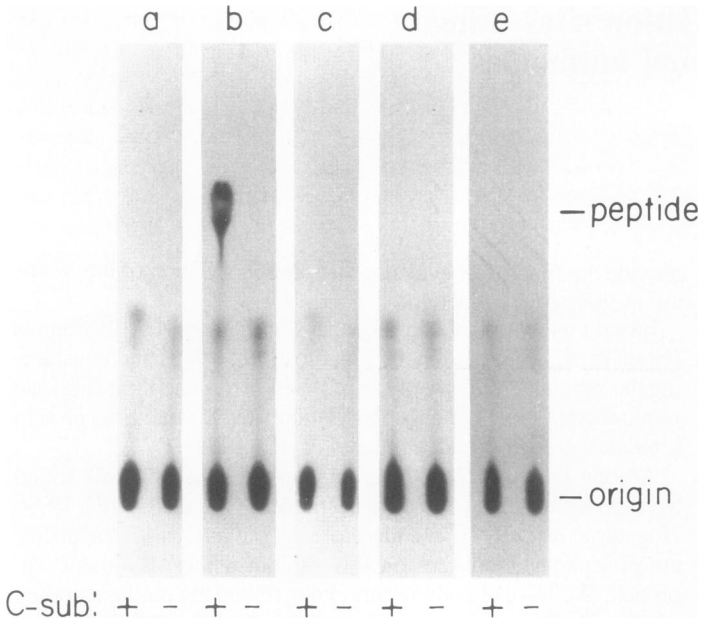

Fig. 2. An autoradiogram of [γ-^{32}P]ATP phosphorylated peptides, separated by thin layer chromatography (t.l.c.). Peptides 364–374 (**a**), 354–367 (**b**) and 373–387 (**c**) of the receptor δ subunit and peptide 330–340 from the α subunit (**d**) were incubated with or without the C-sub of cAMP-dependent protein kinase as described in Materials and methods. For control, reaction mixture in the absence of peptide (**e**) was also applied.

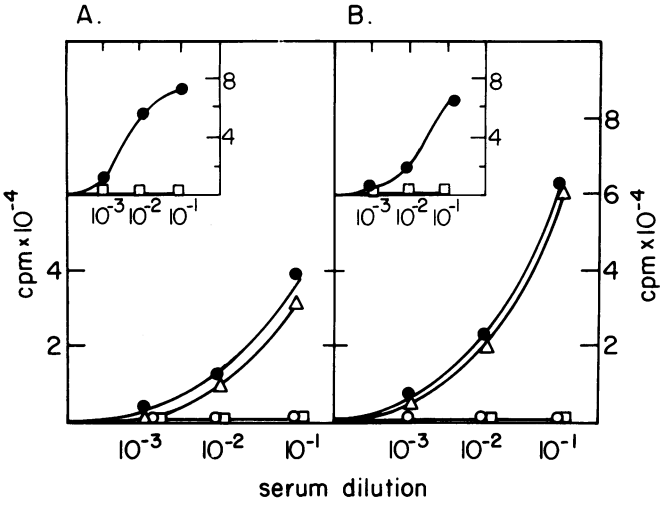

Fig. 3. Antigenic specificity of the anti-peptide antibodies. **(A)** Binding of anti-peptide 364–374 antibodies to AChR in the absence of inhibiting peptide (●) and in the presence of peptide 364–374 (○) or of a control non-related peptide (△). **(B)** Binding of anti-peptide 373–387 antibodies to AChR in the absence of inhibiting peptide (●) and in the presence of peptide 373–387 (○) or of a control non-related peptide (△). The inserts depict the binding of anti-peptide 364–374 **(A)** and 373–387 **(B)** antibodies to the respective peptides. For control the binding of anti-BSA antibodies (□) is shown.

analysed using t.l.c. As can be seen in Figure 2, only peptide 354–367 was phosphorylated (Figure 2b). Two other peptides representing adjacent sequences including the proposed phosphorylation sites for other kinases, were not phosphorylated (Figure 2a,c). In addition, peptide 330–340 from the α subunit which contains the sequence Lys-Arg-Ala-Ser did not undergo phosphorylation under our experimental conditions (Figure 2d). Ninhydrin staining of the t.l.c. plates revealed that all peptides migrated similarly under these conditions (data not shown).

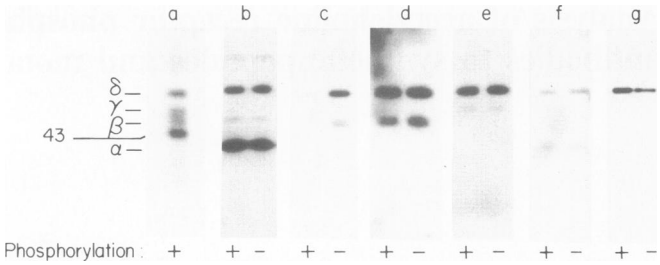

Fig. 4. Binding of anti-peptide antibodies and monoclonal antibodies to phosphorylated and non-phosphorylated receptor. AChR (5 μg) was phosphorylated as described in Materials and methods by the C-sub of bovine heart cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP **(panel a)** or with unlabelled ATP **(panels b–g)**. Phosphorylated receptor (in the presence or absence of enzyme as indicated) was electrophoresed on SDS–polyacrylamide gel (10%). The electrophoresed receptor was blotted onto nitrocellulose membrane filter. **Panel a** was exposed to autoradiography directly. **Panel b** was overlayed with anti-AChR antibodies; **panels c–e** were overlayed with anti-peptides 354–367, 364–374 and 373–387 respectively and **panels f** and **g** were overlayed with mcAbs 5.46 and 7F2. All antibodies were used at 1:100 dilution. For quantitation of binding ^{125}I-protein A was applied when rabbit antiserum was used, and ^{125}I-goat anti-mouse immunoglobulins were applied for detection of mcAbs.

Specificity of the anti-peptide antibodies

In order to elicit antibodies, the synthetic peptides were conjugated to bovine serum albumin (BSA) and injected into rabbits. Reactivity of antibodies to peptide 354–367 with the respective peptide and with AChR was reported previously (Souroujon *et al.*, 1986a). Similarly, the antibodies elicited against peptides 364–374 and 373–387 bound in solid phase radioimmunoassay (SphRIA) to the free peptide and to native *Torpedo* AChR (Figure 3). Although both these latter anti-peptide antibodies bound to their respective peptides to the same extent, they differed in their capacity to cross-react with the receptor. The binding of anti-peptide 373–387 antibodies to the receptor (Figure 3B) was higher than that of anti-peptide 364–374 antibodies (Figure 3A), probably reflecting the exposure and/or conformation of these sequences within the receptor molecule. All three anti-peptide antibodies precipitated significantly receptor complexed with ^{125}I-labelled α-bungarotoxin (data not shown). The corresponding peptide entities are thus available for antibody binding and might also be accessible to phosphorylation enzymes.

As the synthetic peptides were injected in their non-phosphorylated form, it was of interest to test whether the anti-peptide antibodies distinguished between phosphorylated and non-phosphorylated forms of the receptor. AChR was phosphorylated by the C-sub of the cAMP-dependent protein kinase either with non-labelled ATP or with [γ-^{32}P]ATP, and electrophoresed. Samples were then blotted onto nitrocellulose membrane filters. The ^{32}P-labelled lane was autoradiographed directly, while other lanes were further used for overlay with antibodies. As shown in Figure 4, all antibodies used in this experiment bound specifically to the δ subunit of the receptor. In some cases, binding to lower mol. wt bands was observed (panels c,d). Antibodies raised against peptide 354–367 bound preferentially to the non-phosphorylated form of the receptor (Figure 4c). Residual binding to the phosphorylated form of the receptor may either reflect non-phosphorylated receptor molecules or antibody binding to determinants which are not involved in phosphorylation. The other two anti-peptide antibodies as well as anti-receptor antibodies, bound equally well to the phosphorylated and non-phosphorylated forms of the receptor (Figure 4b,d and e). In addition, two anti-AChR monoclonal antibodies, mcAbs 5.46 (Souroujon *et al.*, 1986b) and mcAb 7F2, which are specific for the receptor δ sub-

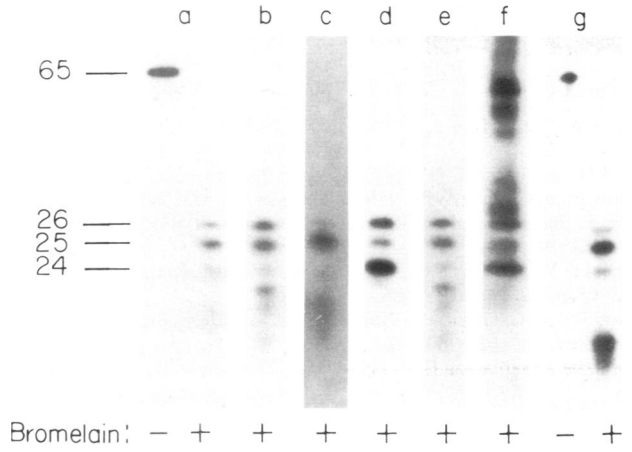

Fig. 5. Mapping of the binding sites of the mcAbs on proteolytic fragments of the δ subunit. AChR (5 μg) was phosphorylated as described in Materials and methods in the presence of [γ-^{32}P]ATP (**g**) or with unlabelled ATP (**a**–**f**). After electrophoresis of AChR on SDS–polyacrylamide gel (10%), the δ subunit was excised from the gel and digested in the stacking gel of SDS–polyacrylamide gel (15%) by bromelain (1 μg/lane) for 1 h and was then further electrophoresed. Digested or non-digested subunits, as indicated, were then blotted onto nitrocellulose membrane filters. The [γ-^{32}P]ATP phosphorylated δ subunit (digested and non-digested) (**g**) was exposed directly. **Panels a**–**c** were overlayed with anti-peptides 364–374, 373–387 and 354–367 respectively. **Panels d** and **e** were overlayed with mcAbs 7F2 and 5.46 respectively. **Panel f** was overlayed with anti RCM-AChR antibodies. ^{125}I-Labelled protein A or ^{125}I-labeled goat anti-mouse Ig were subsequently applied to quantify the binding of rabbit antiserum or mcAbs, as described in Figure 4.

unit were tested for their reactivity with phosphorylated and non-phosphorylated AChR. While mcAb 5.46 reacted equally with phosphorylated and non-phosphorylated receptors (Figure 4f), mcAb 7F2 reacted preferentially with the phosphorylated form (Figure 4g).

Mapping the binding sites of anti-receptor mcAbs

In a previous report (Souroujon *et al.*, 1986a) we have shown that anti-peptide 354–367 antibodies bound to a 25 kd tryptic fragment of AChR. The same fragment also contains the residues which are phosphorylated by the cAMP-dependent protein kinase.

Further analysis aimed at localizing the phosphorylated fragment within the δ subunit was performed in this study by employing antibodies against the other synthetic peptides as well as mcAbs specific for the receptor δ subunit. Phosphorylated δ subunit (either radiolabelled or not) was excised from a 10% polyacrylamide gel, subjected to bromelain digestion and further electrophoresed. The fragments were then blotted onto nitrocellulose membrane filters. The ^{32}P-labelled lanes were autoradiographed directly, while unlabelled lanes were further used for antibody overlay. The results of such an experiment are shown in Figure 5. Bromelain digestion of the δ subunit resulted in a complex array of fragments which were detected by overlay with antibodies to denatured (reduced and carboxymethylated) AChR (RCM-AChR; Bartfeld and Fuchs, 1977; Figure 5f). Only four of these fragments with mol. wts of 26, 25, 24 and ~15 kd were found to be phosphorylated (Figure 5g). Anti-peptide antibodies and also the two mcAbs used reacted mainly with the 26, 25 and 24 kd proteolytic fragments.

Discussion

In this study a synthetic approach was employed in an attempt to localized the phosphorylation sites on the AChR molecule. We have synthesized three peptides which include sequences corresponding to three proposed sites of phosphorylation on the receptor δ subunit (Huganir *et al.*, 1984). Specific phosphorylation of peptide 354–367 by the C-sub of cAMP-dependent protein kinase is demonstrated (Figure 2). This peptide contains the sequence Arg-Arg-Ser-Ser which fulfills the substrate requirements of the enzyme (Kemp *et al.*, 1977). The two other synthetic peptides both containing serine residues, were not phosphorylated. Furthermore, a peptide corresponding to residues 330–340 of the α subunit (Souroujon *et al.*, 1986b) which contains the sequence Lys-Arg-Ala-Ser was not phosphorylated under the experimental conditions used. This result is in agreement with the observation that both endogenous and exogenous cAMP-dependent protein kinases phosphorylate the receptor γ and δ subunits but not its α subunit (Huganir and Greengard, 1983). In a previous report we showed that antibodies elicited against peptide 354–367 specifically inhibit the cAMP-dependent phosphorylation of the δ subunit (Souroujon *et al.*, 1986a). The demonstration of specific phosphorylation of peptide 354–367 further verifies the localization of the cAMP-dependent phosphorylation site on the δ subunit.

In an attempt to analyse the effect of phosphorylation by cAMP-dependent protein kinase on the structure of the receptor, antibodies raised against all three peptides were tested for their binding to the receptor in its phosphorylated and non-phosphorylated forms (Figure 4). Two anti-peptide antibodies directed against the proposed tyrosine-specific phosphorylation site and protein kinase C phosphorylation site, as well as anti-receptor antibodies bound equally to the two forms of the receptor. However, anti-peptide 354–367 antibodies bound preferentially to the non-phosphorylated form of the receptor. As binding to the phosphorylated receptor is significantly reduced, it is assumed that most antibodies are directed against the serine-containing determinant in its non-phosphorylated form and that phosphorylation of this serine prevents antibody binding. The observation that cAMP-dependent phosphorylation does not affect the binding of the two other anti-peptide antibodies implies that no significant conformational changes occur in this region upon phosphorylation. Monoclonal antibody 5.46 which was previously reported to react with posvitin (Pizzighella *et al.*, 1983), suggesting that it is directed against a phosphorylated determinant, reacted equally with both forms of the receptor. On the other hand, mcAb 7F2 was shown to bind preferentially to the receptor in its phosphorylated form, indicating that this antibody is directed against a determinant involved in cAMP-dependent phosphorylation of the receptor δ subunit. Anti-peptide 354–367 antibodies and mcAb 7F2 thus provide powerful tools in the analysis of cAMP-dependent phosphorylation states of the receptor in different experimental and physiological conditions.

We have shown that following proteolytic digestion of the δ subunit all anti-peptide antibodies bound to the same fragments which contain the phosphorylated residues obtained following reaction with the C-sub of cAMP-dependent protein kinase (Figure 5). This was expected since all three peptides are located within a 30-amino acid stretch of the receptor molecule. Mapping the unknown antigenic determinants to which the anti-δ mcAbs are directed was feasible employing anti-peptide antibodies corresponding to selected regions on the receptor δ subunit. Using this approach we were able to demonstrate that the binding sites of the mcAbs reside on the same fragments which contain the sequences corresponding to the synthetic peptides. These results further support the prediction that mcAbs 7F2 and 5.46 are directed against determinants involved in receptor phosphorylation. While mcAb 7F2 is probably directed against a determin-

ant involved in cAMP-dependent phosphorylation of the receptor δ subunit, 5.46 might be directed against a determinant involved in phosphorylation by a different kinase. In view of the potential physiological significance of AChR phosphorylation, all anti-peptide antibodies as well as the two mcAbs presented in this study are currently being used to map other phosphorylation sites on the receptor δ subunit and to elucidate the role of phosphorylation in different functions of the AChR.

Materials and methods

Proteins and peptides

AChR was purified from the electric organ of *Torpedo californica* as described previously (Aharonov *et al.*, 1977). In all experiments affinity-purified AChR was employed.

Peptide synthesis was carried out by the solid-phase method of Merrifield (1965) as previously described (Neumann *et al.*, 1984). The amino acid composition of the peptides was verified by amino acid analysis. The peptides were conjugated to BSA (Sigma) as described (Neumann *et al.*, 1985; Muller *et al.*, 1982).

Immunization procedure

Rabbits were immunized by multisite intradermal injections of 1 mg of conjugate in 0.5 ml of PBS (0.14 M NaCl/0.01 M phosphate buffer, pH 7.2), emulsified in 0.75 ml of complete Freund's adjuvant. A booster injection (0.5 mg of conjugate in complete Freund's adjuvant) was similarly administered 4 weeks later. Further booster injections were given in incomplete Freund's adjuvant. Total rabbit serum was used.

Production of mcAbs

Monoclonal antibody 5.46 against *Torpedo* AChR was elicited as previously described (Souroujon *et al.*, 1983). Monoclonal antibody 7F2 was elicited in a separate hybridization under similar conditions. Both mcAbs were obtained by immunization with intact AChR.

Immunological analysis of antisera

The binding of the antisera to the synthetic peptide and to AChR was analysed by SphRIA (Mochly-Rosen and Fuchs, 1981). The plates were coated either directly with affinity-purified AChR or coated with the peptide by use of glutaraldehyde (Suter, 1982). In the inhibition experiments, the antisera were incubated with peptide (2 μg) for 1 h at 37°C prior to the addition of the antibodies to the plate.

^{125}I-Labelled *Staphylococcus aureus* protein A, radiolabelled by the Bolton–Hunter reagent (Bolton and Hunter, 1972), was applied for quantitation of binding. Electrophoresis of AChR and immunoblotting were performed as previously described (Neumann *et al.*, 1985). The δ subunit was excised from the gel and digested with bromelain (Sigma 1 μg/slot) according to Cleveland *et al.* (1977). Transfer of the fragments and overlay with antibodies was performed as described (Gershoni *et al.*, 1983; Neumann *et al.*, 1985).

Phosphorylation assays

Affinity-purified AChR (5 μg) was incubated in 20 mM Tris–HCl, pH 7.4/20 mM $MgCl_2$/1 mM EDTA/1 mM EGTA/10 mM 2-mercaptoethanol/0.1% Triton X-100/100 μM [γ-^{32}P]ATP (10^2 c.p.m./pmol) with 0.05 μg (25 nM) purified catalytic subunit of bovine heart cAMP-dependent protein kinase (Sigma) in a final volume of 50 μl. Phosphorylation was initiated by addition of [γ-^{32}P]ATP and performed at 30°C for 30 min. The reaction was stopped by addition of 12.5 μl of 8% SDS/0.25 mM Tris–HCl, pH 6.8/40% glycerol (w/v)/8% 2-mercaptoethanol/0.1% bromophenol blue.

Synthetic peptides (1–10 μg) were incubated in 25 mM Tris–HCl, pH 7.4/6 mM $MgCl_2$ and 180 μM [γ-^{32}P]ATP (10^2 c.p.m./pmol) with 0.05 μg (25 nM) purified catalytic subunit of bovine heart cAMP-dependent protein kinase (Sigma) in a final volume of 50 μl. The reaction was initiated by the addition of [γ-^{32}P]ATP and performed at 30°C for 10 min. The reaction was stopped on ice and immediately subjected to t.l.c.

T.l.c.

Phosphorylation products were loaded on silica plates (60F254, Merck) and chromatographed for 4 h at room temperature using as a solvent ethyl acetate: acetic acid:pyridine:water in a ratio of 5:5:1:3. The silica plates were then dried, developed with 1% ninhydrin in acetone to detect all peptides and subjected to autoradiography to identify the ^{32}P-labelled peptides.

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